

Eur. J. Biochem. 220, 893-900 (1994)  
© FEBS 1994

ask. 6

## Translocation and processing of various human parathyroid hormone peptides in *Escherichia coli* are differentially affected by protein-A-signal-sequence mutations

B. Najma KAREEM<sup>1</sup>, Erik ROKKONES<sup>1</sup>, Anders HØGSET<sup>2</sup>, Erik HOLMGREN<sup>1</sup> and Kaare M. GAUTVIK<sup>1</sup>

<sup>1</sup> Institute of Medical Biochemistry, University of Oslo, Oslo, Norway

<sup>2</sup> Nycomed BIOREG AS Oslo, Oslo, Norway

<sup>3</sup> Kabi Pharmacia AB, Sweden

(Received November 23, 1993) – EJB 93 1739/2

Two staphylococcal protein-A signal sequences were constructed and tested for function in *Escherichia coli*, after being linked to human parathyroid hormone (hPTH) cDNAs representing the intact form (1–84 amino acids) and two N-terminal (1–37 and 1–7 amino acids) peptides. One signal sequence was identical to the wild type, and the other signal contained a deletion of 12 bp at the 3' end. The truncated hPTH cDNAs were fused at their 3' ends to IgG-binding domains (ZZ) derived from protein A in order to facilitate purification and characterization. The expression plasmid pSPTH, containing the wild-type signal sequence, secreted efficiently the intact recombinant hPTH (1–84) into the medium. Plasmids containing the truncated hPTH genes after the wild-type signal, gave rise to hPTH–ZZ hybrid proteins which were correctly processed at the N-terminal, but the major fractions appeared in the periplasmic compartment. In contrast, the plasmid pS'PTH which harboured the 4-amino-acid signal deletion did not promote a uniform secretion of intact hPTH (1–84) to the medium, but released a non-processed form both into the periplasmic compartment and to the medium. The related plasmids pS'PTH37ZZ and pS'PTH7ZZ with the mutated signal sequence gave rise to small or trace amounts of unprocessed forms of fusion proteins in the medium and periplasm, thus the secretion competence was markedly reduced. Thus, for correct N-terminal processing, we conclude that the amino acid sequence in the signal adjacent to the expressed protein, is a key determinant. However, release into the medium or periplasmic space appeared to be dependent also on protein folding, irrespective of signal-sequence cleavage. Furthermore, we observed that the peptides with the wild-type signal sequence and correct N-terminal processing, were the only forms that showed internal cleavage of hPTH. Uncleaved signals may contribute to folding characteristics of the ensuing protein and e.g., prevent internal proteolysis.

The molecular mechanism of protein export in Gram-negative bacteria is still poorly understood, although several specific and independent pathways appear to be used for the export of different proteins in a variety of such organisms (for a review see reference [1]).

Secretory proteins are usually synthesized as precursors containing an extra N-terminal extension termed the signal peptide [2–4]. During the translocation process the signal peptide is cleaved off at a specific site by a signal peptidase. In *Escherichia coli* the signal peptidases are located to the outside of the inner membrane [4] cleaving proteins that are secreted to the periplasmic space. Translocation of proteins through the outer membrane to the extracellular medium is

very infrequent in this organism [5–7]. However, cases have been reported where heterologous proteins fused to signal sequences are transported to the growth medium [8, 9]. The mechanism for such translocation is at present largely unknown, as is the possible role of different signal peptide motifs.

In this work we compared the effects of two different signal sequences for translocation of three heterologous peptides that contained the N-terminal part of human parathyroid hormone (hPTH). When the natural signal sequence, which gave rise to a predominant medium localization of correctly processed hPTH(1–84), was mutated, a non-processed form was still released both to the medium and periplasmic space. In comparison, two truncated forms of hPTH cDNA, being C-terminally extended with a part of protein-A-coding DNA (ZZ, [10]), were correctly processed N-terminally if the wild-type signal was used, but appeared now mainly with a periplasmic localization. However, with the mutated signal, even if proteins had an increased intracellular accumulation, they appeared unprocessed in the medium and periplasmic space. In addition to these observations, we found that the internal proteolytic cleavage pattern of the fusion proteins was dependent on whether N-terminal processing occurred.

Correspondence to B. N. Kareem, Institute of Medical Biochemistry, University of Oslo, P. O. Box 1112 Blindern, N-0317 Oslo 3, Norway

Fax: +47 22 851058.

Abbreviations: hPTH, human parathyroid hormone; ZZ, tandem synthetic IgG-binding domains from Staphylococcal protein A; hPTH(1–37)–ZZ, hPTH(1–7)–ZZ, human parathyroid hormone containing the first 37 and the first 7 amino acids of human parathyroid hormone, respectively, fused to ZZ.

Best Available Copy

## MATERIALS AND METHODS

### Materials

Restriction enzymes and other DNA-metabolizing enzymes were from New England Biolabs and were used according to the supplier's recommendations.  $^{125}$ I-Antibodies were from Amersham Corp., and N-terminal-specific anti-PTH antibody was bought from CHIMICON. Synthetic hPTH(1-84) and hPTH(1-34) were from Sigma. Protein A (ZZ) and anti-protein A serum were gifts from KabiPharmacia. Immobilon Poly(vinylidene difluoride) transfer membranes were purchased from Millipore, non-fat dried milk was from Nestlé and molecular-mass standards were from Bio-Rad.

### Bacterial strains, plasmids, and recombinant DNA methods

*E. coli* strains DH5, JM103, HB101 and BJ5183 [11] (obtained from Dr F. Lacroux, Centre de Genetique Molculaire du CNRS, Gif-sur-Yvette, France) were used as bacterial hosts.

Cloning of hPTH cDNA and the subcloning into plasmid pSPTH has been described previously [12, 13].

If not otherwise stated, recombinant DNA methods were performed according to Sambrook et al. [14].

DNA sequencing was performed on plasmid DNA with Sequenase (United States Biochemical Corporation) according to the supplier's manual. The synthetic linkers, oligomers for hybridization and the primers used for the DNA sequencing (-CCTTTTGTAGACTTGAG- and -TCTGTGAGTGA-AA-) were synthesized with an automated DNA synthesizer (KabiPharmacia AB) as described [15].

### Screening for production of protein A

Screening for production of protein A was performed according to a modified version of the methods published by Helfman and Hughes [16] as described by Karcem et al. [17].

### Purification of hPTH(1-84) and ZZ-containing fusion proteins

Recombinant hPTH(1-84) was concentrated and purified by S-Sepharose chromatography, HPLC and polyacrylamide-gel electrophoresis as described [13, 18]. Fusion proteins were purified from growth medium and cell extracts by affinity chromatography on fast flow IgG Sepharose (Pharmacia) [17]. Protein was quantified by means of the Bio-Rad protein assay system [19, 20].

### Radioimmunoassay

Radioimmunoassay of hPTH was carried out as described [21], using an antiserum reactive against epitopes between amino acids 44 and 68 in hPTH.

### Polyacrylamide-gel electrophoresis and immunoblotting

SDS/PAGE, transfer of proteins to poly(vinylidene difluoride) transfer membranes, and staining of the membranes were performed as described [13, 17].

Antibody probing for hPTH(1-84) was performed as described earlier [13]. For specific immunovisualization of the hPTH part in the hybrid proteins containing ZZ tails, special

care had to be taken to avoid IgG-binding interference of the ZZ part. Thus unspecific binding was blocked by incubating the filter in 3.5% human IgG in NaCl/P<sub>i</sub> (136 mM NaCl, 26 mM KCl, Na<sub>2</sub>HPO<sub>4</sub> 2 H<sub>2</sub>O, 15 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.3, adjusted with HCl) overnight at 37°C. For detection of the hPTH regions the N-terminal-specific rabbit anti-hPTH antibody was used (dilution 1:1000), followed by incubation by an  $^{125}$ I-labelled anti-rabbit (Fab)<sub>2</sub> fragment. Antibody incubations, washes and autoradiography were performed as described by Towbin et al. [23]. For detection of ZZ, an anti-ZZ antibody (KabiPharmacia) was used (at dilution 1:1000), employing  $^{125}$ I-labelled anti-rabbit IgG (from donkey) as the secondary antibody.

### Densitometric scanning

Semi-quantitative results from scanning of autoradiograms were obtained with Bio Image, Millipore system, showing linear increments within the working range used (0.2-20 µg protein). Different amounts of a standard hPTH(1-34) fragment and standard ZZ protein were used for estimation of the recombinant hybrid proteins hPTH(1-37) and hPTH(1-7)-ZZ, respectively.

### Amino acid sequence analysis

N-terminal sequence analysis was performed on HPLC fractions or on protein bands isolated after separation on SDS/PAGE and transfer onto poly(vinylidene difluoride) transfer membranes [24]. Sequences were determined on an Applied Biosystems microsequencer (477 A).

## RESULTS

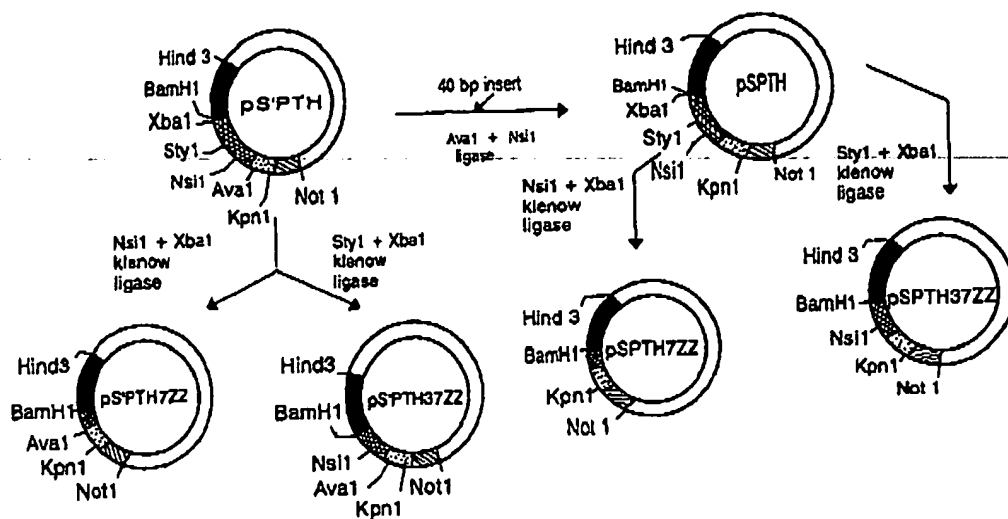
### Expression-plasmid construction

The expression-plasmid construction is outlined in Fig. 1A, and the expressed proteins are given in Fig. 1B. In brief, plasmid pS'PTH with the entire hPTH cDNA (previously referred to as pKP43PTH in [13]), has a four-amino-acid deletion at the C-terminal end of the protein-A signal sequence, placing a Gly in the -1 position and a Pro in the -2 position relative to the first amino acid of hPTH. To obtain the wild-type protein-A signal sequence positioned in front of the hPTH coding sequence, the plasmid pS'PTH was cleaved by *Ava*I/*Nsi*I and a synthetic oligonucleotide,

```
-CCGGCTGCTAACGCTTCTGTGAGTGAAATACAGCTTATATGCA-
-----GACGATTGCGAAGACACTCACTTTATGTCGAATAT-----
```

was inserted between these sites to give the final expression plasmid pSPTH. The plasmids pSPTH37ZZ and pSPTH7ZZ, containing only the 5' 71 bp and 21 bp of hPTH cDNA, respectively, were constructed by removing the *Syl*/XbaI and *Nsi*/XbaI fragments from pSPTH. Plasmids pS'PTH37ZZ and pS'PTH7ZZ were made analogously by excision of the *Syl*/XbaI and *Nsi*/XbaI fragments, from plasmid pS'PTH. Thus, the expression plasmids pSPTH37ZZ, and pS'PTH37ZZ contained hPTH cDNA encoding the first 37 amino acid residues, while pSPTH7ZZ and pS'PTH7ZZ coded for only the first 7 amino acid residues (Fig. 1B). In these constructs the hPTH cDNAs were positioned between DNA coding for the promoter and signal sequence of *Staphylococcus aureus* protein A and the ZZ-part consisting of two IgG-binding domains [10].

A



B

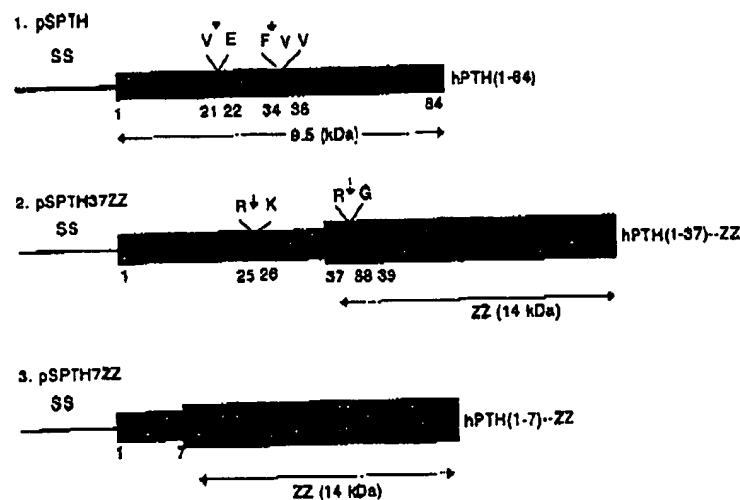


Fig. 1. Schematic drawing of the construction of expression plasmids (A) and the generated fusion proteins (B). (A) Plasmid pS'PTH contained a four-amino-acid deletion at the C-terminal end of the signal sequence. hPTH cDNA sequence (hatched area) was placed between the signal sequence (dotted area) and the tandem IgG-binding domains of *S. aureus* protein A (dark area), and was under the control of the protein A gene promoter (diagonal area). For further details see text. (B) 1, pSPTH and pS'PTH expresses recombinant hPTH(1-84), containing full-length hPTH with the different forms of the signal sequence of staphylococcal protein A (SS) which are also present in the other fusion peptides. 2, pSPTH37ZZ expresses fusion protein hPTH(1-37)-ZZ, where ZZ denotes tandem IgG-binding domains of staphylococcal protein A. 3, pSPTH7ZZ expresses fusion protein hPTH(1-7)-ZZ, where the symbols are as in 2 and the numbers denote the N-terminal amino acids of hPTH. The theoretical sizes of full-length hPTH and ZZ are indicated by the horizontal arrows and the internal proteolytic cleavage sites observed (see text) are indicated by vertical arrows.

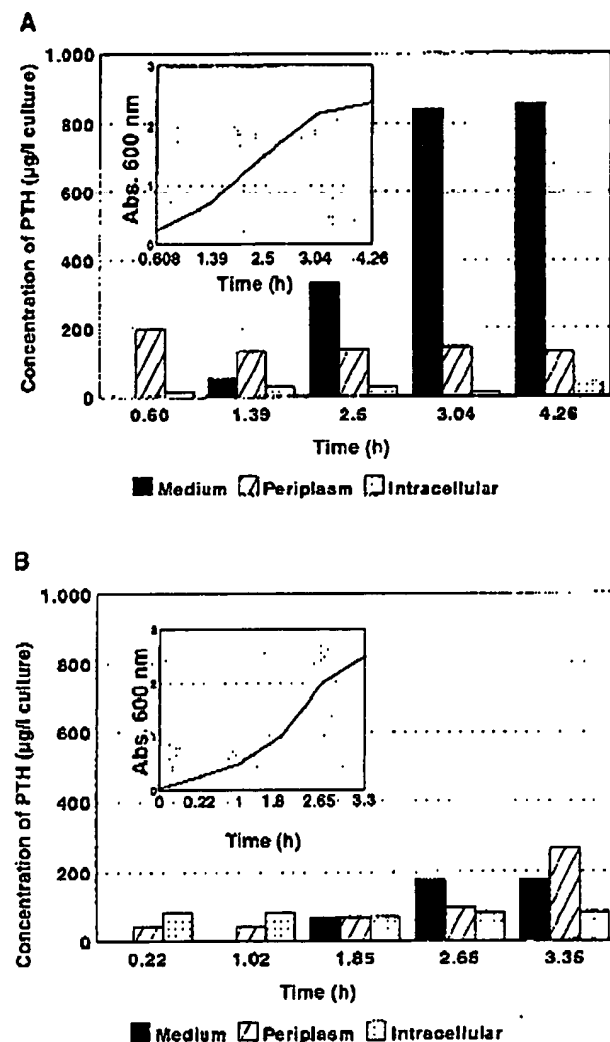
Positive clones transformed with these plasmids were selected by screening for the production of protein A (ZZ) as described above. The correct sequences of the expression plasmids were confirmed by DNA sequencing [25].

Of several *E. coli* strains (JM103, HB101, DH5, BJ5183) tested, BJ5183 gave the highest overall level of expression (data not shown), and this strain was therefore chosen for further studies.

#### Analysis of expressed hPTH forms and their cellular localizations

The production of hPTH in *E. coli* BJ5183 transformed with the plasmids pSPTH and pS'PTH, is shown in Fig. 2. Maximal total production (sum of extracellular, periplasmic and intracellular amounts) of hPTH was about 1 mg/l. It is apparent that the wild-type signal sequence was very efficient

896



**Fig. 2.** Total hPTH production and cellular distribution as a function of culture density. Cells transformed with the expression plasmids pSPTH (A) and pS'PTH (B) were grown as described and the amount of hPTH-immunoreactive material in the intracellular, periplasmic, and medium fractions was determined by radioimmunoassay [21]. The insert shows an increase in culture density [absorbance (Abs.) at 600 nm] as function of time. Mean values that differed by less than 23% from two experiments are shown.

in translocating hPTH to the medium. At later stages of growth about 85% of the product was in the medium while periplasmic localization represented only 12% and the intracellular fraction less than 3% (Fig. 2A). In contrast, the mutated signal sequence caused hPTH to remain partly intracellular (15–20%) and in the periplasm (30–60%), so that only 25–40% was released to the medium (Fig. 2B). With the mutated signal sequence the total amount of hPTH produced was reduced to about 50% (about 0.5 mg/l). The relative distribution and amounts of hPTH obtained with these two different constructs have been summarized in Table 1.

The total amount of IgG-affinity-purified fusion protein expressed by plasmid pSPTH37ZZ as measured by densitometric scanning of immunoblots (using anti-ZZ antibody) was up to about 2.3 mg/l and at least 20-fold greater than

**Table 1.** Cellular distribution profile and production efficiency of hPTH peptides expressed by different expression plasmids. For details, see text. Percentage of total production: +++++, about 85%; ++++, >50%; +++, <40%; ++, about 25%; and +, about 12%.

Plasmid		Distribution of peptide to		
no.	name	medium	periplasm	intracellular
1	pSPTH	+++++	+	(trace)
2	pS'PTH	+(+)	+(+)	+
3	pSPTH37ZZ	(+)	++++	+++
4	pS'PTH37ZZ	(trace)	(trace)	(+)
5	pSPTH7ZZ	++	++++	+
6	pS'PTH7ZZ	(trace)	(+)	(+)

that expressed by the corresponding plasmid pS'PTH37ZZ (0.1 mg/l) (Fig. 3). The cellular distribution of hPTH-ZZ fusion forms observed with these two constructs were distinctly different (Fig. 3, Table 1). The fusion proteins expressed by pSPTH37ZZ were secreted to the periplasmic space (>50%), although a substantial amount (<40%) was also retained intracellularly (Fig. 3A). In contrast, products with the mutated signal sequence (pS'PTH37ZZ) showed a marked translocation block with more than 90% remaining intracellular at peak production (Fig. 3B).

For quantification of IgG-affinity-purified fusion proteins expressed by constructs pSPTH7ZZ and pS'PTH7ZZ, we scanned immunoblots with anti-ZZ serum (Fig. 4). Using pSPTH7ZZ we observed a major localization of the fusion protein (>90% correctly processed) to the periplasmic fraction (50–75%) with a peak production of approximately 1.2 mg/l. About 20–40% of the products appeared in the medium (Fig. 4A). In contrast, expression by pS'PTH7ZZ (Fig. 4) resulted in about 50% of unprocessed hPTH(1–7)-ZZ remaining intracellularly, a corresponding amount being released to the periplasmic space, and only a trace amount appearing in the medium (Fig. 4B). The total amount of fusion protein expressed by the signal-mutated pS'PTH7ZZ was again only about 10% (0.13 mg/l) of that produced by the same gene construct with the wild-type signal (1.2 mg/l; Fig. 4 and Table 1).

In summary, the hPTH-ZZ hybrid forms with the wild-type signal sequence in hPTH(1–37)-ZZ and hPTH(1–7)-ZZ showed an efficient translocation to the periplasmic space and also, to a small degree to the medium (Figs 3A and 4A). The two mutated signal-sequence-fusion-gene constructs demonstrated both a block in secretion and markedly reduced production efficiencies (Figs 3B and 4B).

#### Characterization of the hPTH-related expression products hPTH forms secreted by *E. coli* transformed with pSPTH and pS'PTH

Intact hPTH(1–84) and related secreted forms, were concentrated by S-Sepharose chromatography and purified as described in Materials and Methods. Identification and characterization of the hPTH molecular species were performed by SDS/PAGE and immunoblotting using a mid-region-specific hPTH antiserum, and by amino acid sequence analysis.

In the medium fraction of cells transformed with pSPTH, four major hPTH-related peptides migrating at 13.4, 9.5, 6.0 and 5.5 kDa were observed (Fig. 5A). The major peptide species of 9.5 kDa, migrated with the hPTH standard and,

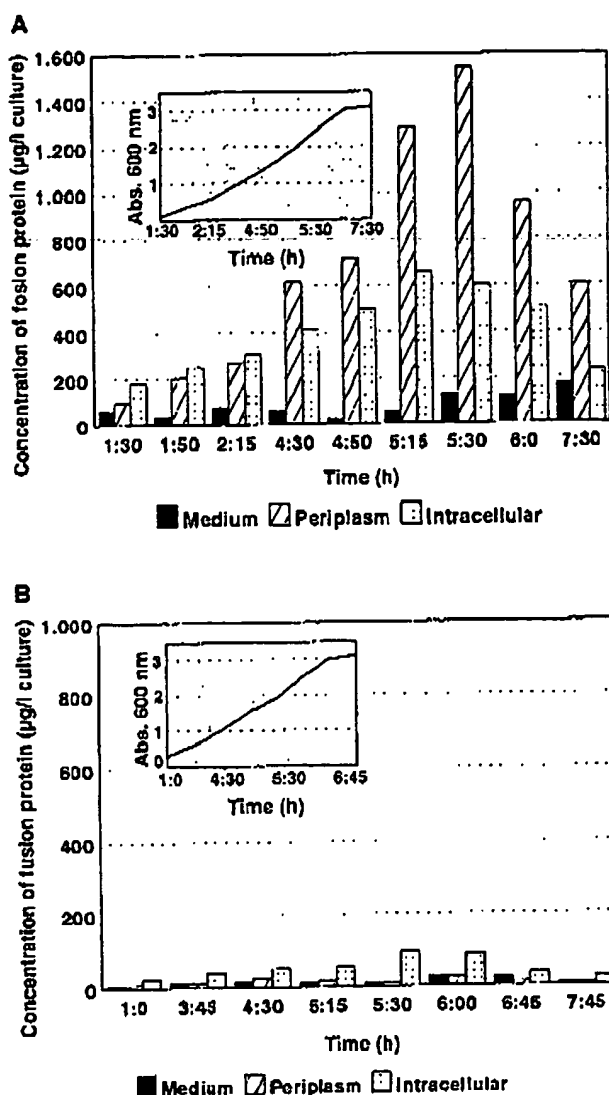


Fig.3. Total production and cellular distribution of hPTH-(1-37)-ZZ-related fusion protein as a function of culture density. Cells transformed with expression plasmids pSPTH37ZZ (A) and pS'PTH37ZZ (B) were grown as described, and the amount of ZZ-containing fusion proteins in the intracellular, periplasmic and medium fractions were determined by scanning immunoblots. The insert shows culture density [absorbance (Abs.) at 600 nm] as a function of time. Results from one of three typical experiments, are shown. The inter-experimental variation was less than 24%.

as judged by amino acid sequence analysis, represented the correctly processed mature hormone. The major degradation product of 6.0 kDa was found to contain the sequence E W L R K K L Q corresponding to amino acids 22-29, thus identifying it as an hPTH C-terminal fragment resulting from proteolytic cleavage between amino acids Val21 and Glu22 (Fig. 1B). The faint band at 13.4 kDa gave both N-terminal and mid-region-specific immunoreaction, but was not characterized further.

Periplasmic fractions of the same culture also revealed four major hPTH-related peptides, migrating at 14.5, 9.5, 6.5 and 6.0-5.5 kDa (Fig. 5A). The largest of these peptides

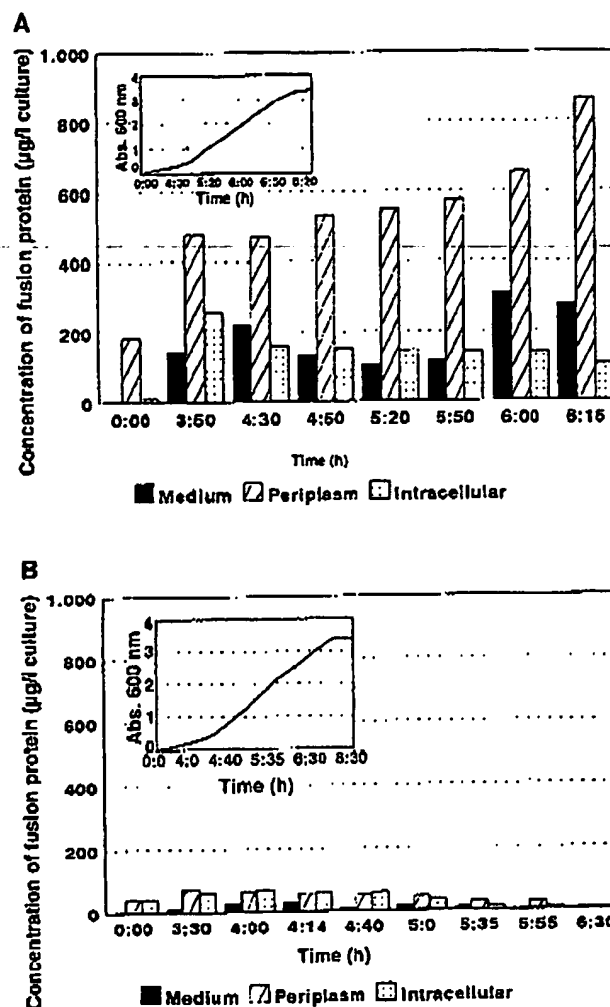
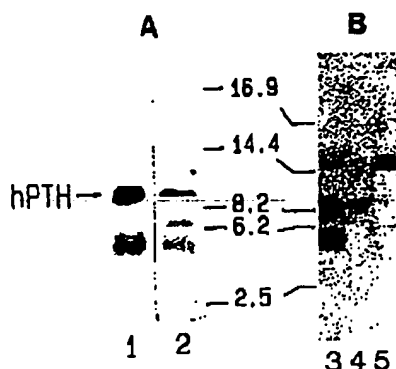


Fig.4. Total production and cellular distribution of hPTH-(1-7)-ZZ fusion protein as a function of culture density. Cells transformed with expression plasmids pSPTH7ZZ (A) and pS'PTH7ZZ (B) were grown as described, and the amount of ZZ-immunoreactive material in the intracellular, periplasmic and medium fractions were determined by scanning immunoblots. The insert shows culture density [absorbance (Abs.) at 600 nm] as a function of time. Data from one of three typical experiments, is shown. The inter-experimental variation was less than 26%.

presumably represent hPTH with an uncleaved signal sequence since we could not resolve the sequence, probably due to blockage of the N-terminal by the translation-initiating *N*-formyl-methionine. The 9.5-kDa polypeptide again represented correctly processed hPTH. Degraded forms were also observed in the periplasm with a major fragment of 5.5 kDa, similar to that appearing in the medium.

In *E. coli* transformed with pS'PTH, the dominating periplasmic hPTH form was a protein of 14.5 kDa migrating with the higher-molecular-mass species produced by pSPTH and probably representing hPTH with an uncleaved signal sequence (Fig. 5B). Attempts to sequence this band proved futile, probably due to N-terminal blockage (see above). The faint band at 6.2 kDa was not characterized further (Fig. 5).



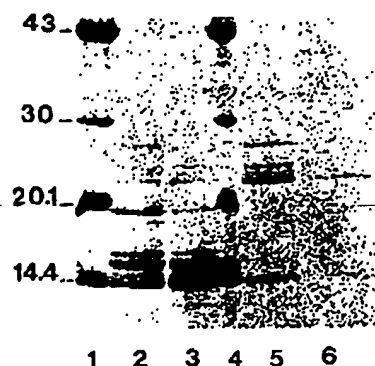
**Fig. 5.** Immunoblot analysis of hPTH secreted by exponentially growing *E. coli* transformed with pSPTH and pS'PTH. From exponentially growing cells the medium and periplasmic fractions were prepared as described. The fractions from 1 l culture were concentrated by freeze-drying and dissolved in 100  $\mu$ l loading buffer, and different amounts were applied on the gel. The relative positions of molecular mass standards are indicated. (A) 4-days-exposed autoradiograph. Lane 1, medium (5  $\mu$ l) and; lane 2, periplasmic fraction (5  $\mu$ l) from cells transformed with pSPTH. (B) Two-weeks exposed autoradiograph. Lane 3, periplasmic fraction (30  $\mu$ l) from cells transformed with pS'PTH included for comparison; lane 4, hPTH standard (0.250  $\mu$ g); lane 5, periplasmic fraction (100  $\mu$ l) from cells transformed with pS'PTH.

#### Analysis of hPTH-ZZ hybrid proteins in different cellular compartments

Medium and periplasmic fractions from cells containing the plasmids pSPTH37ZZ, pSPTH7ZZ, pS'PTH37ZZ and pS'PTH7ZZ were collected in the late-exponential growth phase and the ZZ-containing proteins were purified by IgG-affinity chromatography, analyzed by SDS/PAGE and visualized by Coomassie staining (Figs 6A and 7) and by immunoblotting using an N-terminal-specific antiserum (data not shown). These fusion proteins were further identified by amino acid sequence analysis.

Samples from the medium of cells containing pSPTH37ZZ showed two major ZZ hybrid proteins migrating at 14 kDa and 15 kDa, and a minor species of 15.5 kDa. In addition two fainter bands migrating at 19 kDa and 22 kDa could also be observed (Fig. 6A). The periplasmic fraction from the same cell culture also contained a stronger band at 19 kDa (Fig. 6A). In both lanes 2 and 3 of Fig. 6A we also observed three very faint bands migrating in the 24–28-kDa range. Immunoblotting of a parallel filter showed a main periplasmic immunoreactive band migrating at 19 kDa, the theoretically estimated value for the hPTH(1–37)-ZZ polypeptide (data not shown). Amino acid sequence analysis confirmed this protein to be correctly processed hPTH(1–37)-ZZ. The sequence obtained for the 15-kDa band, X K L Q V H N F, revealed it to be a C-terminal fragment of hPTH(1–37)-ZZ where proteolytic cleavage had occurred between Arg25 and Lys26 (Fig. 1B). The sequence X X X Q H N E A V N N K F obtained for the 14-kDa band corresponded to amino acids +40 to +53, i.e. in the ZZ part of the fusion protein, with cleavage occurring between Arg39 and Gly40 (Fig. 1B).

For cells transformed with pS'PTH37ZZ a totally different migration pattern was observed (Fig. 6). A major medium-fraction band migrating at 24 kDa corresponded to PTH(1–37)-ZZ with an uncleaved sequence as judged by



**Fig. 6.** SDS/PAGE analyses of secreted hPTH-related peptides from *E. coli* transformed with pSPTH37ZZ and pS'PTH37ZZ. From exponentially growing cells the medium and periplasmic fractions were prepared as described. Secreted proteins were purified by IgG-affinity chromatography. The eluates from the IgG-affinity purification were lyophilized, the pellets from 1-l cultures were dissolved in 100  $\mu$ l of the loading buffer [22]. Secreted peptides were analysed by Coomassie staining. Lanes 1 and 4 represent molecular-mass standards. Lanes 2 and 3 represent periplasmic and medium fractions, respectively, from cells transformed with pSPTH37ZZ (applied amount 2.0  $\mu$ l; 2% of the total culture); lanes 5 and 6 contain medium and periplasmic fractions from cells transformed with pS'PTH37ZZ (60  $\mu$ l applied, representing 60% of the culture).



**Fig. 7.** Analysis of secreted hPTH species from *E. coli* transformed with pSPTH7ZZ and pS'PTH7ZZ. The hPTH-ZZ fusion products found in the medium and periplasm were compared after harvesting of exponentially growing cells, and preparation as described. The proteins were purified using IgG-affinity columns. The eluates from the IgG-affinity purification were lyophilized, the pellets were dissolved in 100  $\mu$ l loading buffer and applied to a 15% SDS/polyacrylamide gel. The peptides and protein markers were visualized by Coomassie staining. Lane 1, molecular-mass standards. Lane 2, ZZ standard (0.75  $\mu$ g). Lanes 3 and 4, medium and periplasmic fractions from cells transformed with pSPTH7ZZ (2  $\mu$ l applied, representing 2% of the culture). Lanes 5 and 6, medium and periplasmic fractions from cells transformed with pS'PTH7ZZ (40  $\mu$ l applied, representing 25% of the culture).

SDS/PAGE mobility (Fig. 6) and N-terminal-specific hPTH immunoreactivity (data not shown). We could not sequence the 24-kDa band, probably due to the same reason as stated above. Fainter bands were observed migrating at 14, 25, 27 and 29 kDa. The periplasmic fraction contained fainter bands migrating at 24 kDa and 14 kDa (Fig. 6).

Finally, hybrid proteins produced by cells transformed with pSPTH7ZZ and pS'PTH7ZZ were compared (Fig. 7). The predominant protein expressed by pSPTH7ZZ (migrating at 15 kDa) seemed to have been efficiently processed and secreted to the periplasm (Fig. 7), negligible amounts were seen both in the intracellular fraction and in the medium. In the sequence obtained, S V S E I Q L T R G S X L, amino acids 1–7 represent hPTH, while 8–13 correspond to the N-

terminal part of ZZ. However, cells transformed with pS'PTH7ZZ, expressed a barely visible 15-kDa band in the medium fraction (Figs 7 and 1B). However, two bands were observed at 18 kDa and 15 kDa (Fig. 7) in the periplasmic fraction. The 18-kDa band could represent an unprocessed form of hybrid protein, since again we were not able to sequence this band.

## DISCUSSION

*Escherichia coli* normally secretes very few proteins to the growth medium [26] and the general molecular mechanisms for such secretion are largely unknown. Although the protein-A signal sequence works well in *E. coli* in itself it does not generally translocate proteins to the growth medium, since the natural protein A, as well as a fusion to alkaline phosphatase, translocates only to the periplasmic space [27]. Our results show that a large fraction (more than 85%) of the immunoreactive hPTH is secreted to the growth medium when using the plasmid (pSPTH) with the wild-type protein-A signal sequence placed in front of hPTH(1-84) cDNA.

In comparison, the fusion proteins which are expressed by pSPTH37ZZ and pSPTH7ZZ where the protein A moiety is localized at the 3' end of hPTH cDNA mainly accumulate in the periplasmic space. However, these smaller hPTH fragments which are linked to the ZZ portion are also, to a certain degree released to the medium, provided that a correct N-terminal cleavage occurs. These results demonstrate an interesting and unexpected, differential response to staphylococcal protein-A signal sequence regarding secretion of full-length hPTH compared to the truncated fusion forms hPTH(1-37)-ZZ and hPTH(1-7)-ZZ. Compared to the full-length hPTH(1-84) the translocation of the truncated fusion forms to the medium was less efficient. The results regarding the intact hPTH is also consistent with a possible significance of N-terminal sequence for efficient translocation to the medium provided that the signal is correctly processed.

Works by Inouye et al. [28], and others [29, 30] employing site-directed mutagenesis have indicated that structural features other than the preferred sequences around the cleavage site may play an important role in determining signal-sequence cleavage, e.g., the folding influence of the signal peptide on the secreted protein [28, 30-33]. It has been suggested [34] that, the ensuing translocation of the nascent polypeptide to the periplasm is dependent on the removal of the cleaved protein from the signal-sequence/peptidase complex, results that are consistent with our findings using the two pS'PTHZZ constructs. However, the direct demonstration that uncleaved signal sequence in the pS'PTH construct is translocation competent, indicates that other mechanisms are also involved.

The mutated signal sequence in front of the truncated hPTH with a ZZ tail results in a minimal release to the periplasmic compartment, probably due to secretion incompetence. In comparison, the increased intracellular accumulation of correctly processed hPTH(1-37)-ZZ using the wild-type signal in pSPTH37ZZ may primarily be due to high expression levels exceeding the secretory capacity of the *E. coli* host.

The preservation of the signal sequence in the proteins expressed by plasmids pS'PTH, pS'PTH37ZZ and pS'PTH7ZZ are mainly in accordance with the rules of von

Heijne [3] regarding signal cleavage, since the appearance of a proline residue at position -2 can be expected to inhibit processing at the normal site [35] (before the first amino acid of hPTH). There is a potential cleavage site also at position +3 of hPTH, but this site is apparently not used. Unlike the signal sequence of hPTH [34] the mutated signal sequence of protein A is however able to translocate the unprocessed hPTH to the periplasmic space and medium. This indicates that the hPTH prepro part might contain structures that in some way may act as a membrane anchor in *E. coli*.

A general finding irrespective of the constructs employed, is that if the signal peptide is not cleaved off the ensuing protein, a relatively larger fraction localizes inside the cells. Recent analyses [36] have suggested that some secreted polypeptides may become secretion incompetent if allowed to fold into a native-like conformation prior to membrane translocation.

Not only does it appear that processing and secretion are interdependent phenomena, but also that degradation is at least partly a function of processing. The unprocessed form of the fusion protein somehow avoids such proteolytic attack, as if the presence of the uncleaved signal sequence induces folding which makes these sites inaccessible to proteases.

In conclusion, these results and the model constructs employed have given novel information regarding factors and mechanisms responsible for the translocation of proteins through the inner and outer membranes of *E. coli* and represent a promising basis for further evaluation regarding the impact of protein folding as a discriminant factor in protein export from prokaryotic cells.

The sequence analysis was performed by Dr Knut Sletten, Institute of Biochemistry, University of Oslo. B. N. K. has received economic support from The Norwegian Research Council for Science and the Humanities (NAVF) P. no. 316.90/040. K. M. G. has received economic support from Anders Jahres Foundation for promotion of science, and Rakheh and Otto Kr. Bruuns legacy, Oslo, Norway, and also from Pedersen and Sønn, Oslo, Norway.

## REFERENCES

1. Pugsley, A. P. (1988) Protein secretion across the outer membrane of Gram-negative bacteria, in *Protein transfer and organelle biogenesis* (Das, R. C. & Robbins P. W., eds) pp. 607-652, Academic Press, Inc., Orlando.
2. Wu, H. C. (1986) Proteolytic processing of signal peptides, in *Protein compartmentalization* (Strauss, A. W., Bolme, I. & Krell, G., eds) pp. 33-60. Springer-Verlag, New York.
3. von Heijne, G. (1984) How signal sequences maintain cleavage specificity, *J. Mol. Biol.* 173, 243-251.
4. Donald, O. (1985) *Annu. Rev. Microbiol.* 39, 615-648.
5. Mackman, N. & Holland, B. (1984) Functional characterization of a cloned haemolysin determinant from *Escherichia coli* of human origin, encoding information for secretion of a 107 K polypeptide, *Mol. & Gen. Genet.* 193, 312-315.
6. Muller, D., Hughes, C. & Goebel, W. (1983) Relationship between plasmid and chromosomal hemolysin determinants of *Escherichia coli*, *J. Bacteriol.* 153, 846-851.
7. Holland, B., Blight, M. A. & Kenny, B. (1990) The mechanism of secretion of hemolysin and other polypeptides from gram negative bacteria, *J. Bacteriol. Biotechnol.* 22, 473-491.
8. d'Enfert, C., Chapon, C. & Pugsley, A. P. (1987) Export and secretion of the lipoprotein pullulanase by *Klebsiella pneumoniae*, *Mol. Microbiol.* 1, 107-116.
9. d'Enfert, C., Ryter, A. & Pugsley, A. P. (1987) Cloning and expression in *Escherichia coli* of the *Klebsiella pneumoniae* for production, surface localization and secretion of the lipoprotein pullulanase, *EMBO J.* 6, 3531-3538.

10. Løfdahl, S., Guss, B., Uhlen, M., Philipson, L. & Lindberg, M. (1983) Gene for Staphylococcal protein A. *Proc. Natl Acad. Sci. USA* 80, 697-701.
11. Losson, R. & Lacroute, F. (1981) Cloning of a eukaryotic regulatory gene. *Mol. & Gen. Genet.* 184, 394-399.
12. Høgset, A., Blingsmo, O. R., Gautvik, V. T., Sæther, O., Jacobsen, P. B., Gordeladze, J. O., Alestrøm, P. & Gautvik, K. M. (1990) Expression of human parathyroid hormone in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* 166, 50-60.
13. Høgset, A., Blingsmo, O. R., Sæther, O., Gautvik, V. T., Holmgren, E., Hartmanis, M., Josephson, S., Gabrielson, O. S., Gordeladze, J. O., Alestrøm, P. & Gautvik, K. M. (1990) Expression and characterization of a recombinant human parathyroid hormone produced as a secreted protein in *Escherichia coli* employing the Staphylococcal protein A promoter and signal sequence. *J. Biol. Chem.* 265, 7338-7344.
14. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular cloning: A laboratory manual*, 2nd edn, Cold Spring Harbor Laboratory, Cold Spring Harbor NY.
15. Elmblad, A., Josephson, S. & Palm, G. (1982) Synthesis of mixed oligodeoxynucleotides following the solid phase method. *Nucleic Acids Res.* 10, 3291-3301.
16. Helfman, D. M. & Hughes, H. S. (1987) Use of antibodies to screen cDNA expression libraries prepared in plasmid vectors. *Methods Enzymol.* 152, 451-457.
17. Kureem, B. N., Høgset, A., Holmgren, E. & Gautvik, K. M. (1992) A method for the evaluation of the efficiency of single sequences for secretion and correct N-terminal processing of human parathyroid hormone produced in *Escherichia coli*. *Anal. Biochem.* 204, 26-33.
18. Olstad, O. K., Reppe, S., Gabrielsen, O. S., Hartmanis, M., Blingsmo, O. R., Gautvik, V. T., Haflan, A. K., Christensen, T. B., Øyen, B. T. & Gautvik, K. M. (1992) Isolation and characterization of two biologically active O-glycosylated forms of human hormone produced in *Saccharomyces cerevisiae*: Identification of a new motif for O-glycosylation. *Eur. J. Biochem.* 205, 311-319.
19. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* 72, 248-254.
20. Spector, T. (1978) Refinement of the coomassie blue method of protein quantitation. A simple and linear spectrophotometric assay for less than or equal to 0.5 to 50 microgram of protein. *Anal. Biochem.* 86, 142-146.
21. Gautvik, K. M., Teig, V., Halvorsen, J. F., Arnesen, E., Myhre, I., Helman, P. & Tollman, R. (1979) Development of sequence specific radioimmuno assay of human parathyroid hormone and its use in the diagnosis of hyperparathyroidism. *Scand. J. Clin. Lab. Invest.* 39, 469-478.
22. Lammli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.
23. Towbin, H., Staehelin, T. & Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl Acad. Sci. USA* 76, 4350-4354.
24. Matsudaira, P. (1987) Sequence from picomole quantities of protein electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* 262, 10035-10038.
25. Chen, E. Y., Seeburg, P. H. (1985) Supercoil sequencing: a fast and simple method for sequencing plasmid DNA. *DNA (NY)* 4, 165-170.
26. Inouye, S., Duffaud, G. & Inouye, M. (1986) Structural requirement at the cleavage site for efficient processing of the lipoprotein secretory precursor of *Escherichia coli*. *J. Biol. Chem.* 261, 10970-10975.
27. Folz, R. J. & Gordon, J. I. (1987) The effects of deleting the propeptide from human preproapolipoprotein A-1 on co-translational translocation and signal peptidase processing. *J. Biol. Chem.* 262, 17221-17230.
28. Duffaud, G. & Inouye, M. (1983) Signal peptidase recognizes a structural feature at the cleavage site of secretory protein. *J. Biol. Chem.* 258, 10224-10228.
29. Pluckhun, A. & Knowles, J. R. (1987) The consequences of stepwise deletions from the signal processing site of beta-lactamase. *J. Biol. Chem.* 262, 3951-3957.
30. Fikes, J. D. & Bassford, P. J. Jr. (1987) Export of unprocessed precursor maltose-binding protein to the periplasm of *Escherichia coli* cells. *J. Bacteriol.* 169, 2352-2359.
31. Fikes, J. D., Barkocy-Gallagher, G. A., Klapper, D. G. & Bassford, P. J. Jr. (1990) Maturation of *Escherichia coli* maltose-binding protein by signal peptidase 1 *in vivo*. Sequence requirements for efficient processing and demonstration of an alternate cleavage site. *J. Biol. Chem.* 265, 3417-3423.
32. Luijck, J., High, S., Wood, H., Giner, A., Tollervey, D. & Dobberstein, B. (1992) Signal sequence recognition by an *Escherichia coli* ribonucleoprotein complex. *Nature* 359, 741-743.
33. Nilsson, I. & von Heijne, G. (1992) A signal peptide with a proline next to the cleavage site inhibits leader peptidase when present in a sec-independent protein. *FEBS Lett.* 299, 243-246.
34. Born, W., Freeman, M., Hendy, G. N., Rapoport, A., Rich, A., Potts, J. T. & Kronenberg, H. M. (1987) Human preproparathyroid hormone synthesized in *Escherichia coli* is transported to the surface of the bacterial inner membrane but not processed to the mature hormone. *Mol. Endocrinol.* 1, 5-14.
35. Nilsson, I. & von Heijne, G. (1992) A signal peptide with a proline next to the cleavage site inhibits peptidase when present in a sec-independent protein. *FEBS Lett.* 299, 243-246.
36. Nilsson, B., Marks, B. C., Kintz, I. D. & Anderson, S. (1991) Secretion incompetence of bovine pancreatic trypsin inhibitor expressed in *Escherichia coli*. *J. Biol. Chem.* 266, 2970-2977.



**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**